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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*
Leuven Research & Development V.Z.W.
Groot Begijnhof
Benedenstraat 59A
B-3000 Leuven
Belgium

Patents ADP number *(if you know it)* 56885700 1

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4. Title of the invention
Ligands for use in therapeutic compounds for the treatment od hemostasis disorders

5. Name of your agent *(if you have one)* William E. Bird of Bird Goën & Co

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LIGANDS FOR USE IN THERAPEUTIC COMPOSITIONS FOR THE TREATMENT OF HEMOSTASIS DISORDERS.

FIELD OF THE INVENTION

The present invention relates to novel cell lines and to ligands, namely human and/or humanized monoclonal antibodies, as well as fragments such as Fab, Fab', $F(ab')_2$, scFv, single variable domains, complementarity determining regions and derivatives and combinations thereof, obtainable from the said cell lines. It also relates to pharmaceutical compositions comprising said ligands and to methods of preventing and treating coagulation disorders and resulting thrombotic pathologic conditions in humans by administration of the said ligands to patients in need thereof. It also relates to methods of obtaining specific mammalian antibodies.

BACKGROUND OF THE INVENTION

The coagulation of blood involves a cascading series of reactions leading to the formation of fibrin. The coagulation cascade consists of two overlapping pathways required for hemostasis. The intrinsic pathway comprises protein factors present in circulating blood, while the extrinsic pathway requires tissue factor which is expressed on the cell surface of a variety of tissues in response to vascular injury. Agents interfering with the coagulation cascade, such as heparin and coumarin derivatives, have well known therapeutic uses in the prophylaxis of venous thrombosis.

Aspirin also provides a protective effect against thrombosis. It induces a long-lasting functional defect in platelets, detectable clinically as a prolongation of the bleeding time, through inhibition of the cyclooxygenase activity of the human platelet enzyme prostaglandin H-synthase (PGHS-1) with doses as low as 30 to 75 mg. Since gastrointestinal side effects of aspirin appear to be dose-dependent, and for secondary prevention, treatment with aspirin is recommended for an indefinite period, there are practical reasons to choose the lowest effective dose. Further it has been speculated that a low dose (30 mg daily) might be more antithrombotic but attempts to identify the optimal dosage have yielded conflicting results. It has been claimed that the dose of aspirin needed to suppress fully platelet aggregation may be higher in patients with

cerebrovascular disease than in healthy subjects and may vary from time to time in the same patient. However, aspirin in any daily dose of 30 mg or higher reduces the risk of major vascular events by 20 % at most, which leaves much room for improvement.

Further, the inhibiting role of aspirin may lead to prevention of thrombosis as well as to excess bleeding. The balance between the two depends critically on the absolute thrombotic versus hemorrhage risk of the patient.

In patients with acute myocardial infarction, reduction of infarct size, preservation of ventricular function and reduction in mortality has been demonstrated with various thrombolytic agents. However these agents still have significant shortcomings, including the need for large therapeutic doses, limited fibrin specificity, and significant associated bleeding tendency. Recombinant tissue plasminogen activator (t-PA) restores complete patency in just over one half of patients, whereas streptokinase achieves this goal in less than one third. Further, reocclusion after thrombolytic therapy occurs in 5 to 10 % of cases during the hospital stay and in up to 30 % within the first year according to Verheugt et al., *J. Am.Coll.Cardiol.* (1996) 27:618-627. Thus numerous studies have examined the effects of adjunctive antithrombin therapy for patients with acute myocardial infarction. As an example, U.S.Patent 5,589,173 discloses a method for dissolving and preventing reformation of an occluding thrombus comprising administering a tissue factor protein antagonist, which may be a monoclonal or polyclonal antibody, in adjunction to a thrombolytic agent.

One approach to blocking platelet aggregation involves monoclonal antibodies specific for GPIIb/IIIa receptors. Specifically, a murine monoclonal antibody named 7 E3 said to be useful in the treatment of human thrombotic diseases is described in EP-A-206,532 and U.S.Patent 5,387,413. However it is known in the art that murine antibodies have characteristics which may severely limit their use in human therapy. As foreign proteins, they may elicit an anti-immunoglobulin response termed human anti-mouse antibody (HAMA) that reduces or destroys their therapeutic efficacy and/or provokes allergic or hypersensitivity reactions in patients, as taught by Jaffers et al., *Transplantation* (1986) 41:572. The need for readministration in therapies of thromboembolic disorders increases the likelihood of such immune reactions. While the

use of human monoclonal antibodies would address this limitation, it has proven difficult to generate large amounts of such antibodies by conventional hybridoma technology.

Recombinant technology has therefore been used to construct "humanized" antibodies that maintain the high binding affinity of murine monoclonal antibodies but exhibit reduced immunogenicity in humans. In particular, there have been suggested chimeric antibodies in which the variable region (V) of a non-human antibody is combined with the constant (C) region of a human antibody. As an example, the murine Fc fragment was removed from 7 E3 and replaced by the human constant immunoglobulin G Fab region to form a chimera known as c7 E3 Fab or abciximab. Methods of obtaining such chimerical immunoglobulins is described in detail in U.S.Patent 5,770,198.

The potential for synergism between GPIIb/IIIa inhibition by monoclonal antibody 7 E3 Fab and thrombolytic therapy was evaluated by Kleiman et al., *J.Am.Coll.Cardiol* (1993) 22:381-389. Major bleeding was frequent in this study. Hence, the potential for life-threatening bleeding is clearly a major concern with this combination of powerful antithrombotic compounds.

In a recent attempt to reduce the immunogenicity of murine antibodies, only the complementarity determining region (CDR), i.e. regions of hypervariability in the V regions, rather than the entire V domain, are transplanted to a human antibody. Such humanized antibodies are known as CDR-grafted antibodies. Such one CDR-grafted antibody was successfully constructed against the relatively simple nitrophenacetyl antigen, however the construction of CDR-grafted antibodies recognizing more complex antigens has resulted in antibodies having binding activity significantly lower than the native non-human antibodies. In numerous cases it was demonstrated that the mere introduction of non-human CDRs into a human antibody is insufficient to maintain full binding activity. While a refined computer model of the murine antibody of interest is required in order to identify critical amino-acids to be considered in the design of a humanized antibody, and general theoretical guidelines were proposed for such design, in all cases the procedure must be tailored and optimized for the particular non-human antibody of interest.

Tissue factor (TF), being a membrane glycoprotein functioning as a receptor for factor VII and VIIa and thereby initiating the said extrinsic pathway, has been investigated as a target for anticoagulant therapy. In addition to this role, TF has been implicated in pathogenic conditions such as vascular disease and gram-negative septic shock.. A study attempting to characterize the anticoagulant potential of murine monoclonal antibodies showed that the inhibition of TF function by most of the monoclonal antibodies assessed was dependent upon the dissociation of the TF/VIIa complex that is rapidly formed when TF contacts plasma. One monoclonal antibody, TF8-5G9, was capable of inhibiting the TF/VIIa complex without dissociation of the complex, thus providing an immediate anticoagulant effect in plasma, as disclosed in WO 96/40,921.

Targeted clotting factors exhibit both a medium molecular weight range (about 45,000 to 160,000) and a relatively high normal plasma concentration (at least 0.01 micromol/L).

One persistent concern with all available thrombolytic and anti-thrombotic agents, including aspirin, is to induce a risk of overdose and therefore of excessive and life-threatening bleeding.

Thus, there is a need in the art for efficient compounds for the treatment of coagulation disorders, which are free from the above-mentioned bleeding problem. In particular for such compounds based on humanized antibodies, there is a need for compounds with full anticoagulant activity but free from the well known immunogenicity problem.

In the efforts to meet these needs, very little attention has been paid to factor VIII, probably because of its low plasma concentration and because of the complex interactions between factor VIII, von Willebrand factor (vWF) and phospholipids. The precise mechanism of action of specific antibodies is far from being elucidated. The link between factor VIII and hemophilia A has discouraged scientists from using it as a therapeutic solution fearing that it may lead to bleeding problems.

Factor VIII is a protein providing coagulant cofactor activity and is one of human clotting factors with a rather high molecular weight (265,000) and a very low normal plasma concentration (0.0007 micromol./litre). With its 2,332 amino-acid

residues, factor VIII is one of the longest known polypeptide chains and is synthesized in the liver, the spleen and the placenta. Its gene has been shown to include 186,000 nucleotides.

Factor VIII circulates as inactive plasma protein. Factors V and VIII are homologous proteins sharing a common structural configuration of triplicated A domains and duplicated C domains with structurally divergent B domains connecting the A2 and A3 domains. Factor VIII circulates in a multiplicity of fragmented species in a tightly associated complex with von Willebrand factor at a concentration of 1 nmol/L. Factor VIII activation occurs by a cleavage between the A1 and A2 domains, resulting in the unstable heterotrimeric factor VIIIa molecule. Factor VIIIa binds tightly to membranes that contain acidic phospholipids. Factor VIII contains a phospholipid binding site in the C2 domain, between amino-acids 2302 and 2332, according to Arai et al. in *J.Clin.Invest.* (1989) 83:1978. Within the same factor VIII region, there is also a von Willebrand factor binding site acting in conjunction with amino-acid residues 1645-1689 in the A3 domain according to Shima et al. in *Throm.Haemost.* (1993) 69:240 and *J.Biol. Chem.* (1994) 269:11601.

Antibodies inhibiting the co-factor activity of factor VIII are classified as type I or type II inhibitors according to their capacity to inhibit factor VIII either completely (type I) or only partially (type II). According to Gawryl et al., *Blood* (1982) 60:1103-9, the reduced inactivation of factor VIII by type II inhibitors is believed to be due to a steric effect of von Willebrand factor (vWF) and data indicate that type II inhibitors react with different antigenic determinants than type I antibodies and that these determinants are partially blocked in the factor VIII/vWF complex. Biggs et al., *Br.J.Haematol.* (1972) 23:137 previously provided an alternative interpretation, however derived as well from data obtained by using human polyclonal antibodies, that a type II inhibitory pattern could be related to low affinity.

European patent applications EP-A-123,945, EP-A-152,746 and EP-A-432,134 all disclose monoclonal antibodies produced by hybridoma cell lines and having a specific reactivity pattern with factor VIIIc polypeptide fragments. These monoclonal antibodies are said to be useful for detecting the presence of factor VIIIc and related polypeptides in plasma by immunoassay techniques, but their therapeutic potential is not

suggested. in these documents.

A study has reported an association of raised factor VIII activity and von Willebrand factor (vWF) levels with ischemic heart disease (IHD). The apparent low incidence of IHD in hemophilia is in agreement with this finding. Factor VIII activity and vWF levels are higher in those patients with deep vein thrombosis than in controls.

Among compounds with anti-thrombotic properties other than aspirin, it was shown by Maraganore et al., *Circulation* (1992) 86:413 that a synthetic 12-aminoacid peptide corresponding to residues 1675-1686 in factor VIII inhibits cleavage by thrombin of the heavy chain required for the activation of the procoagulant activity of factor VIII and also of the light chain required to dissociate factor VIII from von Willebrand factor and that tyrosine sulfation of said peptide potentiates its recognition by factor VIII.

J.Clin.Invest. (1988) 82:206-211 describes obtaining an animal model for hemophilia A by infusion of human anti-factor VIII antibody in rabbits. According to WO 95/01570, antibodies against the light chain of human or porcine factor VIIIc were produced in a first animal and subsequently a temporary hemophilia was induced in a second animal by means of the purified monospecific antibody obtained. U.S.Patent 5,804,159 also discloses inducing a temporary clotting disorder in a mammal by means of an anti-plasma antibody preparation acting on several blood coagulation factors, e.g. a preparation comprising antibodies against human von Willebrand factor and factor VIII, or against factor VIII/von Willebrand factor-complex, or against procoagulants, anticoagulants, clot structure factors, fibrinolysis factors and phospholipids.

Further, Jacquemin et al. in *Blood* (1998) 92:496-506 refers to a factor VIII-specific human IgG4 monoclonal antibody (BO2C11) produced by a cell line derived from the memory B-cell repertoire of a hemophilia A patient with inhibitors. BO2C11 is said to recognize the C2 domain of factor VIII and to inhibit its binding to both vWF and phospholipids. It is said to completely inhibit the procoagulant activity of native and activated factor VIII with a specific activity of 7,000 Bethesda units/mg.

However, none of the above-mentioned compounds involving factor VIII was described as a therapeutic means. In fact there is a technical prejudice among those skilled in the art against investigating anti-factor VIII antibodies for anti-thrombotic

therapy because it is assumed that, a deficiency in factor VIII being the result of hemophilia A, such antibodies would induce a bleeding state.

The present invention is based on overcoming this technical prejudice in the art and on the surprising determination of new ligands, namely new human and humanized monoclonal antibodies and fragments, derivatives and homologs thereof. These may exhibit an unforeseen "plateau effect", i.e. the achievement of only a partial inactivation of a factor involved in hemostasis, in particular in the coagulation cascade, either individually or in combination. The ligands may bind to a factor or a complex of factors resulting in only partially impairing the function of a physiologically functional site of the said factor or factor complex. This makes the ligands particularly suitable for treating coagulation disorders and resulting thrombotic pathologic conditions while minimizing the risk of bleeding. Particularly useful is a property of ligands in accordance with the present invention to allow some physiological function of the affected site even when the ligand is in molar excess. The ligands may be anti-factor VIII antibodies or antibodies against a factor VIII complex, in particular human or human hybrid monoclonal antibodies which bind to factor VIII or a factor VIII complex and at least partially inhibit the activity of factor VIII.

SUMMARY OF THE INVENTION

The present invention is related to new ligands, namely new monoclonal human or humanized antibodies and fragments and derivatives and homologs thereof which bind to a factor in hemostasis, in particular to a factor or factors of the coagulation cascade and more in particular bind to factor VIII or a complex thereof; to polypeptides or other molecules which bind to a factor or factors in hemostasis; to a novel cell line from which said monoclonal antibodies may be obtained; to pharmaceutical compositions comprising said ligands and to methods of prevention and treatment of coagulation disorders and resulting thrombotic pathologic conditions in humans by the administration of said ligands to patients in need thereof.

An object of the present invention is to provide an effective anti-thrombotic therapy which reduces the risk of bleeding in animals, particularly humans.

It is a further object to provide therapeutic compositions which provide an

effective anti-thrombotic therapy which reduces the risk of bleeding in animals, particularly humans.

It is still a further object of the present invention to provide an antithrombotic therapy and antithrombotic therapeutic compounds which are safer to use than known therapies and compositions.

One aspect of the present invention is to target a human protein factor involved in hemostasis, in particular in the coagulation cascade, more in particular factor VIII or a complex thereof, using specific ligands. Preferably, these ligands only partially inhibit the function of the targeted factor so that a residual activity of the factor remains even when the ligand is in a molar excess. A curve may be established of the inhibiting effect of ligands in accordance with the present invention with respect to a factor against concentration of the ligand and the concentration determined at which a minimal residual factor activity still exists which is at least 1%. The residual factor activity at five times this concentration should not be substantially different from the residual activity at the minimal point. It is especially a further aspect of the present invention to provide high affinity monoclonal antibodies, both human and humanized, as well as fragments, derivatives, and homologs of any of these, having the capacity to only partially inactivate a factor or factors in hemostasis, in particular in the coagulation cascade and more in particular factor VIII or a complex thereof, even in molar excess of the ligand, thereby preventing the risk of overdosage and the resulting bleeding complications. It is still another aspect of the present invention to provide a novel cell line producing the respective human monoclonal antibody.

The present invention also includes polynucleotide sequences which encode for the antibodies or fragments thereof mentioned above. It will be appreciated that a multitude of nucleotide sequences exist which fall under the scope of the present invention as a result of the redundancy in the genetic code. The present invention also includes complementary sequences which bind to the antibodies or fragments thereof mentioned above. In particular, the present invention includes probes constructed from the antibodies or fragments thereof mentioned above or from the polynucleotides or from the complementary sequences mentioned above.

The present invention further provides a method of attenuation of coagulation in

humans, comprising administering a ligand such as a monoclonal antibody, either human or humanized, or fragment or derivative or homolog thereof capable of partially inactivating a factor or factors in hemostasis, in particular in the coagulation cascade and more in particular factor VIII or a complex thereof to a patient in need of such attenuation even when the ligand is in a molar excess. It further provides a method of treatment or prevention of a thrombotic pathologic condition in humans, comprising administering a ligand such as a monoclonal antibody, either human or humanized, or fragment or derivative or homolog thereof capable of partially inactivating a factor or factors in hemostasis, in particular in the coagulation cascade and more in particular factor VIII or a factor VIII complex to a patient in need of such treatment or prevention even when the ligand is in a molar excess. In a preferred embodiment, the thrombotic pathologic condition is selected from intravascular coagulation, arterial thrombosis, arterial restenosis, venous thrombosis and arteriosclerosis.

Another embodiment of the present invention is directed to a pharmaceutical composition comprising a ligand having the capacity of binding to a site on a factor or factors in hemostasis, in particular in the coagulation cascade, and more in particular factor VIII or a complex thereof, for only partially inactivating the said factor or factor complex even when the ligand is in molar excess, in admixture with a pharmaceutically acceptable carrier. The said ligand preferably is a high affinity anti-factor VIII or anti-factor VIII complex monoclonal antibody, either human or humanized, or hybridized or fragment or derivative or homolog thereof. The pharmaceutical composition of the invention may further comprise a therapeutically effective amount of a thrombolytic agent.

Another embodiment of the present invention is directed to the selection of specific monoclonal antibodies. The conventional technique of immunizing an animal such as a mouse with a protein such as factor VIII illicits an immunological response which may involve several epitopes on the factor VIII molecule. The present invention provides a more selective method of obtaining specific antibodies against an epitope of a wild-type protein. A donor is provided, e.g. a mammal such as a human, which has an at least partly functional modified version of the wild-type protein. The modification may be due to any cause, e.g. race or variety, to genetic defects at birth, to an illness or

by human interference, e.g. immunotolerance against the functionally modified version. The modification lies in the domain of interest. The donor is then administered a sufficient quantity of the wild-type protein (e.g. factor VIII) until an immune response is generated. Selection of B-cells from the donor will result in a greater chance of obtaining a monoclonal antibody against an epitope in the region of the modification.

The present invention will now be described with reference to the following drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 presents the results of producing human monoclonal antibodies derived from a hemophilia A patient, expressed in the form of IgG antibody binding to factor VIII in ELISA.

Fig. 2 provides a graph showing inhibition of factor VIII activity by the monoclonal antibody BO C211.

Fig. 3 provides a graph showing inhibition of factor VIII activity by the monoclonal antibody produced from cell line KRIX 1.

Fig. 4 provides a graph showing inhibition of the binding of activated factor VIII on phosphatidyl-L-serine by the monoclonal antibody produced from cell line KRIX 1.

Fig. 5 provide graphs showing the influence of certain polyclonal antibodies on the dissociation of activated factor VIII from von Willebrand factor.

Figs. 6 and 8 show amino acid sequences (the lower lines) and nucleotide sequences (upper lines) for the variable regions V_H of the short chains of BO 2C11 and the KRIX 1 monoclonal antibodies, respectively. Also shown are the three CDR's of each chain which are each an individual polypeptide ligand in accordance with an individual embodiment of the present invention.

Figs. 7 and 9 show amino acid sequences (the lower lines) and nucleotide sequences (upper lines) for the variable regions V_L of the long chains of BO 2C11 and the KRIX 1 monoclonal antibodies, respectively. Also shown are the three CDR's of each chain which are each an individual polypeptide ligand in accordance with an individual embodiment of the present invention.

DEFINITIONS

The term "antibody" refers to intact molecules as well as fragments thereof, such as Fab, Fab', $F(ab')_2$, Fv which are capable of binding to the epitope determinant of the relevant factor or domain of the factor.

"Humanized antibody" as used herein, refers to antibody molecules in which amino acids have been replaced in the non-antigen binding regions in order to more closely resemble a human antibody.

A "Reshaped human antibody" or a "Human hybrid antibody" as used herein, refers to a human antibody in which amino acids in the antigen binding regions of a human antibody have been replaced with sequences in accordance with the present invention, e.g. CDR's, or other parts of variable regions which have been derived from the repertoire of human antibodies.

The term "homology" or "homologous" as used herein with reference to ligands in accordance with the present invention refers to a molecule which will compete with or inhibit binding of one of the ligands in accordance with the present invention to the target site. The binding should be specific, i.e. the binding of the alternative molecule should be as specific to the site as the ligand in accordance with the present invention. Where the ligands in accordance with the present invention include amino acid sequences, homology may include having at least 80%, more preferably 90% and most preferably 95% amino acid sequence identity with the relevant ligand.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will be described with reference to certain embodiments and to certain figures but the present invention is not limited thereto but only by the claims. In particular, the present invention will mainly be described with reference to ligands to factor VIII but the present invention is not limited thereto. The present invention relates to a general concept of partially inactivating a factor in hemostasis by the selection of certain antibodies and by the production of human or humanized monoclonal antibodies or fragments, derivatives or homologs thereof and using these for anti-thrombotic therapy and in anti-thrombotic therapeutic compositions. These

ligands and compositions may have the advantageous property that the inactivation of the factor is only partial even when the ligand is in a molar excess. This means that even though the ligand is used in an amount which might be expected to inactivate completely the targeted factor, the inactivation is still incomplete.

The present invention provides a particular cell line producing human monoclonal antibodies which are reactive with human factor VIII and more specifically have the capacity of inactivating the co-factor activity of human factor VIII by interfering with proteolytic cleavage site or von Willebrand factor or tenase complex reaction or by inducing a three-dimensional conformational change in factor VIII, in particular by targeting a domain of factor VIII and by recognizing epitopes located in the said domain. One preferred domain is the C1 domain of factor VIII but the present invention is not limited thereto. A site on the C2 domain of factor VIII may be partially inhibited. The present invention also includes antibodies which reduce the release rate of factor VIII from von Willebrand factor. These antibodies specifically target factor VIII when bound to von Willebrand factor and hence target an epitope associated with the complex of factor VIII and von Willebrand factor. The present invention also provides fragments of any of the above antibodies such as Fab, Fab', $F(ab')_2$, scFv, CDR's, single variable domains as well as derivatives and homologs and combinations of these. More particularly, these antibodies and fragments may target a domain of factor VIII, in particular the C1 domain of factor VIII. They may partially inhibit a site on the C2 domain of factor VIII. They may also target an epitope associated with the complex of van Willebrand factor and factor VIII. An aspect of the present invention is therefore to provide ligands which bind to a first site (e.g. in the C1 domain of factor VIII) remote from a functional second site (e.g. the site in the C2 domain of factor VIII which is responsible for binding phospholipids) in such a way that the function of the second site is only partially impaired even when the ligand is in a molar and therapeutic excess.

The cell line named KRIX 1 producing monoclonal antibodies was deposited with the BCCM™/LMBP (Belgian Co-ordinated Collections of Microorganisms) plasmid collection laboratorium voor moleculaire biologie, University of Ghent K.L. Ledeganckstraat 35, B-9000 Ghent, BE under accession number LMBP 5089CB on

July 1, 1999.

The present invention further provides cell lines producing monoclonal antibodies having a reactivity substantially similar to that of the human monoclonal antibodies obtained from the above-mentioned deposited cell line, as well as the human monoclonal antibodies obtainable from these further cell lines.

The present invention further provides human or human hybrid ("reshaped") monoclonal antibodies against factor VIII, the antibodies comprising elements derived only from humans. By human hybrid monoclonal antibodies is meant a hybrid antibody constructed from a human antibody and variable regions in accordance with the present invention. Conventionally, it has only been possible to obtain antibodies against factor VIII derived from animals, e.g. mice, or to construct chimeric antibodies from human antibodies with the variable portions derived from mice antibodies.

The present invention also provides ligands having the capacity of only partially inactivating a factor involved in hemostasis. in particular in the coagulation cascade of blood, preferably factor VIII or a complex thereof, by binding to a site of the said factor or complex, the partial inactivation also taking place when the ligand is in a molar excess. The site to which the ligand binds may or may not be directly or substantially involved in a physiological interaction of the said factor or complex. For instance, the ligand may bind to a site which is at a predetermined distance away from a physiologically functional site of the said factor. By partial inactivation, herein we mean an at most 99% inactivation, or an at most 95% inactivation or an at most 90 % inactivation or an at most 80% inactivation as determined by a suitable test method such as the chromogenic assay, available from Coatest™, Kabi Vitrum, Brussels, BE or Chromogenix AB, Mölndal, SE. The level of activation required may depend upon the physiological function of the factor. On the other hand, in order to provide therapeutic usefulness, inactivation of the blood factor should be at least 50 %, preferably at least 60 % and more preferably at least 70 %, as determined by the same test method as above. It will be appreciated that the ligands in accordance with the present invention operate in a different way from the mechanism conventionally ascribed to type II antibodies against factor VIII. One conventional mechanism is that of competition with another factor, e.g. von Willebrand factor. The kinetics of a competition mechanism

mean that if the one species is at a high concentration compared with the other (e.g. in a molar excess), the inhibition is effectively complete. In contrast, the ligands of the present invention reach a plateau in inactivation of the relevant factor, substantially independent of the excess of the ligand. The other conventional mechanism ascribed to type II antibodies is that of low affinity. Also in this case, an excess will drive the reaction to complete inhibition.

When the blood factor targeted is factor VIII, such ligands may be human monoclonal antibodies obtainable from the deposited cell line KRIX 1, preferably being of class IgG, and which have the capacity of only partially inactivating the co-factor activity of factor VIII. More specifically the invention relates, in a preferred embodiment, to human monoclonal antibodies from such origin which are able to recognize epitopes located in the C1 domain of factor VIII. Although the inventors do not wish to be bound to a single explanation or theory, it is believed that the binding of such human monoclonal antibodies results in partial impairment of the binding of activated factor VIII to phospholipids, a necessary step for cofactor activity expression.

The present invention further provides monoclonal antibodies having substantially the same characteristics as above disclosed and being produced by on purpose immunization in animals, preferably in mouse, for instance by injecting human factor VIII in mice and then fusing the spleen lymphocytes with a mouse myeloma cell line, followed by identifying and cloning the cell cultures producing anti-factor VIII antibodies. The monoclonal antibodies produced in animals are then humanized, for instance by associating the binding complementarity determining region ("CDR") from the non-human monoclonal antibody with human framework regions - in particular the constant C region of human gene - such as disclosed by Jones et al. in *Nature* (1986) 321:522 or Riechmann in *Nature* (1988) 332:323.

The present invention also provides fragments and derivatives, in particular complementarity determining regions ("CDR's") of the above monoclonal anti-factor VIII antibodies as well as homologs thereof. For instance, the invention provides antigen-binding fragments Fab, Fab' and $F(ab')_2$ generated by proteolytic digestion of the said monoclonal antibodies using methods well known in the art, such as described by Stanworth et al., *Handbook of Experimental Immunology* (1978), vol.1 chapter 8

(Blackwell Scientific Publications). Such fragments, which contain the antibody binding site, have lost a number of properties of the parent antibody, such as complement activation or capacity to bind to Fc gamma receptors. The present invention also includes scFv, and single variable domain fragments of the antibodies and combination of these fragments and the fragments mentioned above.

The invention also provides soluble or membrane anchored single-chain variable parts of the above monoclonal antibodies. The DNA sequences of the variable parts of human heavy and light chains are amplified in separated reactions and cloned. A 15 amino acid linker sequence, for instance (Gly4 Ser)3, is inserted between VH and VL by a two-steps polymerase chain reaction (PCR, Dieffenbach and Dveksler, "PCR Primer, a laboratory manual", 1995, Cold Spring Harbour Press, Plainview, NY, USA). The resulting fragment is inserted into a suitable vector for expression of single chain fragment variable (scFv) as soluble or phage-displayed polypeptide. This can be achieved by methods well known to those skilled in the art, such as described by Gilliland et al., *Tissue Antigens* (1996) 47:1-20. The present invention also includes a ligand comprising peptides representative of hypervariable regions of a monoclonal antibody which can be obtained by synthesis using an applied biosystem synthesizer, e.g. a polypeptide synthesizer model 9050, Milligen, USA or related technology, which alone or in combination with other or similar hypervariable regions will exert properties similar to that of the parent antibody.

The invention further provides a pharmaceutical composition for the prevention or treatment of disorders of hemostasis, in particular of the coagulation cascade and resulting thrombotic pathologic conditions in humans, comprising a ligand as disclosed hereinabove, in admixture with a pharmaceutically acceptable carrier. More preferably the said monoclonal antibody is a human monoclonal antibody or fragment or derivative or homolog thereof obtainable from cell line KRIX 1 deposited with the Belgian Co-ordinated Collections of Micro-organisms under accession number LMBP 5089CB. The degree of homology is preferably at least 80%, more preferably 90 %, and most preferably 95% and the homology is preferably particularly referenced in respect to the complementarity determining regions. A ligand in accordance with the present invention may also include a synthetic polypeptide of equivalent potency.

The pharmaceutical composition of the present invention may further comprise, in view of the so-called adjunctive anti-thrombotic treatment, a therapeutically effective amount of a thrombolytic agent. Such thrombolytic agents, as well as their usual dosage depending on the class to which they belong, are well known to those skilled in the art. Among numerous examples of thrombolytic agents which may be included in the pharmaceutical compositions of the invention, the following non-limiting list may be particularly cited: T-Pa, streptokinase, reptilase, TNK-t-Pa or staphylokinase.

Suitable pharmaceutical carriers for use in the pharmaceutical compositions of the invention are described for instance in Remington's Pharmaceutical Sciences 16th ed. (1980) and their formulation is well known to those skilled in the art. They include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents (for example phenol, sorbic acid, chlorobutanol), isotonic agents (such as sugars or sodium chloride) and the like. Additional ingredients may be included in order to control the duration of action of the monoclonal antibody active ingredient in the composition. Control release compositions may thus be achieved by selecting appropriate polymer carriers such as for example polyesters, polyamino acids, polyvinyl pyrrolidone, ethylene-vinyl acetate copolymers, methylcellulose, carboxymethylcellulose, protamine sulfate and the like. The rate of drug release and duration of action may also be controlled by incorporating the monoclonal antibody active ingredient into particles, e.g. microcapsules, of a polymeric substance such as hydrogels, polylactic acid, hydroxymethylcellulose, polymethyl methacrylate and the other above-described polymers. Such methods include colloid drug delivery systems like liposomes, microspheres, microemulsions, nanoparticles, nanocapsules and so on. Depending on the route of administration, the pharmaceutical composition comprising the active ingredient may require protective coatings. The pharmaceutical form suitable for injectionable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation thereof. Typical carriers therefore include biocompatible aqueous buffers, ethanol, glycerol, propylene glycol, polyethylene glycol and mixtures thereof.

The present invention also provides the use of a ligand as disclosed above as a medicament. More preferably the medicament used in the present invention is a means

for preventing and/or treating disorders of hemostasis, in particular, coagulation disorders and other thrombotic pathologic conditions in humans. The said ligand may be provided to a patient by means well known in the art. i.e. orally, intranasally, subcutaneously, intramuscularly, intradermally, intravenously, intraarterially, parenterally or by catheterization. According to the present invention, the ligand may be used as a medicament in conjunction or association with a thrombolytic agent such as disclosed hereinabove under the heading of pharmaceutical compositions.

Thus the present invention provides a method of treatment and/or prevention of hemostasis disorders, a thrombotic pathologic condition in a human, as well as a method of attenuation of coagulation in a human, comprising administering to a patient in need of such treatment or prevention or attenuation of coagulation a therapeutically effective amount of a ligand as disclosed hereinabove. Preferably the said ligand is a human or humanized monoclonal antibody obtainable from cell line KRIX 1 deposited with the Belgian Co-ordinated Collections of Micro-organisms under accession number LMBP 5089CB or an antigen-binding fragment Fab, Fab' or $F(ab')_2$, a complementarity determining region (CDR), a soluble or membrane-anchored single-chain variable part (scFv), a single variable domain or a derivative or combination of any of these.

A therapeutically effective amount as used herein means .1 microgram to 5 milligram per kg of body weight, more preferably 1 microgram to 1 milligram per kg of body weight. It will be appreciated that, in view of the long half-life time of most IgG human antibodies, ligands which are monoclonal antibodies of the said class will enjoy a periodicity of treatment which participates in the comfort of the patient.

As preferred embodiments of the said thrombotic pathologic condition to be prevented or treated, there may be cited intravascular coagulation, arterial thrombosis (which may be responsible for acute myocardial infarction and stroke), arterial restenosis, venous thrombosis (which commonly occurs in peripheral veins as a consequence of accidental or surgical trauma or immobilization) or arteriosclerosis. In a most preferred method of treatment, the patient is provided with a bolus (intravenously injected) at a dosage determined by the ordinary skilled physician depending on criteria which establish the particular patient's clinical condition.

The method of treatment and/or prevention according to the invention may

include further treatment or prevention of the same thrombotic pathologic condition by administrating, preferably by sequentially administrating, to the patient a therapeutically effective amount of a thrombolytic agent such as disclosed hereinabove under the heading of pharmaceutical compositions. Sequentially, as used herein, means that the ligand of the present invention and the known thrombolytic agent are administered to the patient sequentially but not simultaneously.

The present invention, as embodied in the various above aspects, has a number of advantages. The major advantage of the therapeutic use of the human monoclonal antibodies of the invention is that the treatment is highly specific for the immune response under consideration. In hypercoagulation states, the specificity of the human monoclonal antibodies of the invention ensures that interaction within the coagulation cascade pathway is limited to the factor recognized by the antibody.

More specifically, the use of the anti-factor VIII antibodies having the above-mentioned preferred characteristics brings a unique combination of the advantages related to the targeting of factor VIII, those related to the characteristics of factor VIII inhibition and those related to the use of antibodies :

- targeting factor VIII means that neutralizing a co-factor activity such as that of factor VIII carries no risk of completely inhibiting the enzymatic activity it enhances, thereby representing an advantage over methods targeting directly enzymes such as factor IX.
- the embodiments of the inhibitors described above have in common that they efficiently but only partially inhibit the co-factor activity of factor VIII, even when the monoclonal antibody of the invention is used in more than 100-fold excess. Monoclonal antibodies in accordance with the present invention achieve a plateau effect in inactivation of factor VIII, thereby avoiding the risk of overdosing the patient in need of anti-thrombotic therapy.
- human IgG antibodies exhibit a prolonged half-life time of three weeks (except for IgG3 which is one week), thus providing very stable plasma levels of the anti-thrombotic agent and allowing for a drastic reduction in the frequency of administration. Further, the use of human antibodies or derivatives carries a minimal risk of inducing immune responses.

The invention is further described by the following examples which are provided for illustrative purposes only.

EXAMPLE 1 - production of monoclonal antibodies derived from hemophilia A patients.

Human monoclonal antibodies of the desired specificity and characteristics are produced by transformation of B lymphocytes obtained from the peripheral blood of patients suffering from hemophilia A or acquired hemophilia. The method of selecting patients is an embodiment of the present invention. In order to illicit a more specific immunological response, patients are sought who have an impaired function of a physiologically active protein, e.g. factor VIII. By "impaired" is meant that some residual function is available but that this is less than is known for the wild-type of the same protein. A comparison between the self-protein and the wild-type protein should exhibit a difference in the two proteins, preferably in a region or domain which is of interest. The difference may be a deletion or a substitution of one or more amino acids with others. The patients are then administered enough of the wild-type protein to illicit an immunological response. Then, B-lymphocytes are extracted from the patients and selected based on the production of antibodies which have desirable properties. Although reference is made to "patients" above, the method in accordance with this embodiment may be applied generally to mammals. The above procedure results in a greater chance of obtaining antibodies which target the domain containing the defect.

B cells are transformed by infection with the Epstein-Barr virus and activation of surface antigens using techniques well known by those skilled in the art. Cell supernatants containing appropriate antibodies are identified by a specific assay procedure such as described in more details hereinbelow.

Thus, antibodies towards factor VIII are identified by reacting the supernatant with polystyrene microtitration plates coated with factor VIII or with factor VIII/von Willebrand factor complexes. The binding of specific antibodies is detected by addition of a non human IgG reagent coupled to an enzyme. Addition of an enzyme substrate which is converted to a colored compound in the presence of the enzyme allows the detection of specific antibodies. Such methods referred to as enzyme-linked

immunoassays (ELISA) are well known to those skilled in the art and described in details e.g. in *Current Protocols in Immunology*, chapter 2, John Wiley & Sons (1994), the content of which is incorporated herein by reference.

More specifically in the present case, the binding of anti-factor VIII IgG antibodies was detected by addition of a horseradish peroxidase labeled mouse monoclonal antibody specific for human Fcγ. The IgG subclass of the anti-factor VIII antibody was detected in ELISA, as presented in Fig. 1. The inhibition of factor VIII functional activity was tested in a functional coagulation assay as follows. Equal volume of cell culture supernatant and of a pool of normal plasma were incubated for two hours at 37°C and the residual factor VIII activity measured thereafter. Those antibodies which significantly inhibit factor VIII activity are shown with an asterisk in Fig. 1.

B cells (such as BO 2C11) producing anti-factor VIII antibodies are then expanded and cloned by limiting dilution as described for instance in *Current Protocols in Immunology* (see supra). Anti-factor VIII antibodies having the capacity to inhibit the procoagulant activity of factor VIII as described above are identified using a chromogenic assay kit such as a factor VIII chromogenic assay assay from Dade, Düchingen, Germany or Coatest™ commecially available from Kabi Vitrum, Brussels, BE, or Chromogenix AB, Mölndal, SE (see Fig. 2). Equal volumes of monoclonal antibodies BO 2C11 and a pool of normal blood plasma were incubated for 2 hours at 37°C. BO 2C11 concentrations before mixing are shown on the X axis. The reduction of factor VIII activity was measured in a coagulation assay and was expressed as a percentage of the activity obtained in the absence of antibody. The residual activity goes to zero asymptotically (complete inhibition).

Antibodies which inhibit factor VIII function with sufficient affinity but do not inhibit factor VIII pro-coagulant activity completely, even when used in large antibody excess, were selected in a further embodiment of the present invention. A representative example of such an antibody is provided in Fig. 3 where, equal volumes of KRIX 1 and of recombinant factor VIII or of normal plasma being incubated for two hours at 37°C and concentrations (expressed in microg/ml) of KRIX 1 before mixing with plasma being as indicated, the residual factor VIII activity was measured using the above-mentioned chromogenic assay. Fig. 3 interestingly shows about 60 % factor VIII

inhibition at a concentration of 0.1 microg/ml and more interestingly an asymptotic factor VIII inhibition of about 80% in the whole range of concentrations from 0.5 to 10 microg/ml.

The thus selected antibodies are then produced in bulk culture and purified by affinity chromatography using methods well known to those skilled in the art.

The details of a non-limiting preparation technique are as follows. Human recombinant factor VIII (specific activity: 4000 IU/mg) was obtained from Hyland (Glendale, Ca) as material for laboratory use only; plasma-derived (pd) fVIII-vWf complex, purified by ion exchange chromatography (specific activity ± 160 IU/mg protein; 15:1 vWf to fVIII w/w ratio), and purified fVIII-depleted vWf (vWf to fVIII w/w ratio 4800:1; lot 951016) were obtained from the Belgian Red Cross (Brussels, Belgium).

Peripheral blood samples were collected from donors suffering mild hemophilia and with inhibitors. The peripheral blood mononuclear cells (PBMC) were immortalized by EBV infection concomitantly to the activation of surface antigens. Four hundred and eighty cell lines were screened by ELISA for production of antibodies towards factor VIII. For example, one cell line, named KRIX 1, was successfully cloned by limiting dilution. Clonality was verified by RT-PCR amplification of mRNA coding for the V regions of the antibody heavy and light chains: a single sequence was obtained from 10 clones of PCR products. Purified antibodies were obtained by passage of KRIX 1 cell culture supernatant on Protein-A Sepharose. An ELISA performed with IgG subclass- and light chain-specific antibodies identified KRIX-1 as an IgG4k.

Human monoclonal antibodies were purified by adsorption on immobilized Protein A (high-TRAP^R Protein A; Pharmacia, Uppsala, Sweden). Fab fragments of human monoclonal antibody were prepared by papain digestion. One mg of a selected antibody was diluted to 500 µg/ml in phosphate buffer (40 mmol/L KH_2PO_4, 60 mM $Na_2HPO_4.2H_2O$, 0.15M NaCl) containing 50 mmol/L L-cystein (Sigma), 1 mmol/L EDTA (Merck) and 10 µg papain (Sigma). The mixture was incubated for 3 h at 37°C with continuous agitation. The reaction was stopped by addition of iodoacetamide to a final concentration of 75 mmol/L for 30 min at RT. The digested antibody was dialysed against phosphate-buffered saline (140 mmol/L NaCl, 67 mmol/L KCl, 20 mmol/L

Na_2HPO_4, 4.4 mmol/L KH_2PO_4, pH 7.4). The undigested IgG and Fc fragments were then eliminated by passage over protein A sepharose (Hi Trap Protein A; Pharmacia). The Fab fragment was further purified by gel filtration chromatography on a Superdex 200 (Pharmacia).

Conventional methods were used for the detection of anti-fVIII IgG antibodies, the determination of IgG subclass, and the evaluation of inhibition of fVIII binding to vWf. For the analysis of the inhibition of the binding of rfVIII to a selected antibody by Fab and native antibody. Maxisorb polystyrene plates (Nunc) were coated for 2 h with 50 µl of the antibody diluted to 5 µg/ml in glycine-buffered saline (20 mmol/L glycine, 34 mmol/L NaCl, pH 9.2). After washing, 50 µl of biotin-labeled rfVIII diluted to 1 µg/ml in Tris-casein (10 mmol/L tris(hydroxymethyl)-aminoethane, pH 7.3, containing 150 mmol/L NaCl and 0.5% casein) were mixed for 1 h at 37°C with 50 µl of human IgG at various dilutions. A 50-µl aliquot of the mixture was added to the plates for 2 h at RT. After washing, the binding of biotinylated rfVIII was detected by sequential addition of avidin-peroxydase and OPD.

rfVIII (final concentration 0.2 µg/mL) was incubated with human IgG antibody at different concentrations for 2 hours at 37°C and the residual fVIII activity was assessed by a chromogenic assay (Coatest™ Factor VIII, Chromogenix AB, Mölndal, Sweden or Kabi Vitrum, Brussels, Belgium). Inhibition of plasma fVIII activity was measured by the Bethesda method, in which a pool of normal plasma collected in buffered trisodium citrate was used as fVIII source. Residual fVIII activity was assessed by a chromogenic or by a one-stage clotting assay.

EXAMPLE 2 - Production of monoclonal antibodies by immunization in animals.

Alternatively, monoclonal antibodies having the same characteristics as disclosed in example 1 can be produced by on purpose immunization in animals. Thus, mice are injected with human factor VIII in Freund's adjuvant

Monoclonal anti-human factor VIII antibodies are then obtained by fusion of spleen lymphocytes with a mouse myeloma cell line. Cell culture supernatants producing anti-factor VIII antibodies are identified and cloned by limiting dilution, using methods described in *Current Protocols in Immunology* (see supra). Further

selection of inhibitors having the desired capacity to inhibit the pro-coagulant activity of factor VIII is carried out as described in example 1.

Monoclonal antibodies produced in mice are then humanized. Thus, sequences of the variable parts of mouse heavy and light chains are aligned with human immunoglobulin variable regions to identify human antibody with the greatest homology in framework regions. The DNA fragment encoding humanized variable regions are then synthesized by a PCR-based CDR (complementarity determining regions) grafting method as described for instance in Sato et al., *Cancer Research* (1993) 53:851-6. The final PCR product coding for the heavy chain variable part of the humanized antibody is digested and subcloned upstream of the human C gamma-1 gene in a first expression plasmid. The humanized light chain variable region of the final construction is inserted upstream of the C kappa gene in a second expression plasmid. The two constructions are then co-transfected into COS cells expression system.

EXAMPLE 3 - Characterization of anti-factor VIII antibodies.

Monoclonal antibodies of either human (example 1) or animal (example 2) origin are characterized using an assay system by which their capacity to inhibit the binding of factor VIII to phospholipids is evaluated. Thus, polystyrene microtitration plates are coated with phosphatidyl-L-serine. Soluble recombinant factor VIII at 2 microg/ml final concentration is mixed for 30 minutes at 37°C with various concentrations of the antibody under evaluation. The mixture is then rapidly activated with thrombin and added to phosphatidyl-L-serine coated plates. The said plates were then incubated for two minutes at 21°C and the binding of factor VIII was detected by addition of the anti-factor VIII A1 domain mAbF14A2, for two minutes, followed by a two minute incubation with HRP-conjugated goat anti-mouse Fcγ. Results of this experiment are shown in Fig. 4 for the monoclonal antibody produced from the cell line KRIX 1. On the figure, the average of activated factor VIII binding in the absence (closed symbols) or presence (open symbols) of the antibody, as well as the standard deviation of triplicates are indicated. Controls in the absence of factor VIII gave OD 490 lower than 0.05. Fig. 4 clearly shows that the monoclonal antibody produced from cell line KRIX 1 inhibits significantly the binding of factor VIII to phospholipids but

brings about incomplete inhibition even when added in large excess.

To demonstrate that in absence of plasma, KRIX 1 did not recognize the mutated fVIII light chain of the donor, DNA fragments encoding wild-type and mutated fVIII light chains were synthesized. The corresponding proteins were expressed in reticulocyte lysates. The correct folding of native and mutated light chains was determined by immunoprecipitation with the human monoclonal antibody BO2C11, which recognizes a conformational epitope within the carboxy-terminal part of the fVIII light chain. Immunoprecipitation experiments indicated that BO2C11 bound wild-type and Arg2150His light chains, whereas KRIX 1 captured exclusively the wild-type light chain. Prolonged exposure of SDS-PAGE gels to the autoradiography film failed to detect any significant binding of KRIX 1 to the mutated light chain. Control experiments showed no binding to assay reagents other than fVIII or fVIII fragments, and preincubation with soluble rfVIII prevented the binding to methionine-labeled fVIII fragments, confirming the binding specificity.

KRIX 1 did not recognize fVIII in Western blotting indicating that the epitope recognized was conformational. Further epitope mapping was therefore performed with fVIII fragments produced in reticulocyte lysates. Preliminary experiments had indicated that such an approach was efficient for the synthesis of fVIII domains. The immunoprecipitation procedure using labeled fVIII domains produced in reticulocyte lysate was validated by mapping the epitope recognized by the human monoclonal antibody BO2C11. A complete agreement was observed between the binding to fVIII C2 deletion fragments produced in reticulocyte lysates and the binding to recombinant fragments produced in *E. coli* or COS cells. KRIX 1 bound to full-length light chain, to fragments corresponding to A3C1, C1C2, and the isolated C1 domain. In contrast, KRIX 1 did not bind to the C1 or C1C2 domains with the substitution Arg2150His, although in a control experiment, the Arg2150His C1C2 domain was bound by BO2C11 like its normal counterpart.

A search was made for other mutations in the light chain which could alter the binding of KRIX 1. As shown in Table I, KRIX 1 inhibited the activity of all mutated fVIII molecules tested so far, except those carrying the mutation Arg2150His.

Table I. Inhibition of FVIII activity in mild hemophilia A patients' plasma.

FVIII mutation	FVIII activity (IU/ml)	KRIX-1 Inhibition (%)
Arg1689Leu	0.04	>70
Arg1749His	0.39	>70
Gly1750Arg	0.38	>70
Ala1824Val	0.16	>70
Asp1825Gly	0.17	>70
His1961Tyr	0.24	>70
Arg1966Gln	0.08	>70
Met2010Ile	0.04	>70
Ser2011Asn	0.23	>70
Val2016Ala	0.05	>70
Asn2019ser	0.13	>70
Leu2052Phe	0.23	>70
Asp2074Gly	0.13	>70
Thr2086Ile	0.08	>70
Ile2098Ser	0.20	>70
Phe2101Leu	0.06	>70
Asn2129Ser	0.20	>70
Arg2150His	0.03	0
Pro2153Gln	0.02	65
Arg2159Leu	0.16	>70
Trp2229Cys	0.02	>70
Gln2246Arg	0.08	>70

In the use of KRIX 1 as a medicament to partially inhibit factor VIII mild mutations of factor VIII will not affect the effectiveness of the therapy.

KRIX 1 inhibited the binding of fVIII to vWf in a dose-dependent manner. The concentration of KRIX 1 required to achieve 50% inhibition (IC_{50}) of fVIII binding was 0.25 µg/mL and more than 95% inhibition was obtained with 20 µg/mL of KRIX 1. Fab fragments of KRIX 1 also inhibited fVIII binding to vWf. However, on a molar basis 15 times more Fab than native KRIX 1 was required to inhibit 50% of fVIII binding to vWf. Additional experiments were performed to exclude that the KRIX 1 Fab fragments still contained intact or partially digested antibody. SDS-PAGE analysis of the Fab fragments purified by protein A adsorption and gel filtration chromatography showed a single band. The presence of trace amounts of Fcγ fragments remaining bound to Fab fragments was excluded in ELISA. Wells with insolubilised fVIII were incubated with native or Fab KRIX 1. Binding of both Fab and native KRIX 1 could be detected by addition of peroxydase labeled anti-k light chain IgG. By contrast, addition of peroxydase labeled anti-Fcγ IgG did not reveal any specific binding even when 100 µg/ml of the Fab preparation were incubated into the wells. By comparison, the addition 0.1 µg/ml of the native antibody gave rise to a significant binding. In ELISA, a 15-fold higher concentration of Fab than of native antibody was required to inhibit 50% of the binding of biotinylated KRIX 1 onto insolubilised fVIII, indicating that the Fab KRIX 1 fragment had a lower affinity for fVIII than the native antibody. Accordingly, the requirement for higher concentrations of KRIX 1 Fab than of native antibody to inhibit fVIII binding to vWf should be attributed to the reduced affinity of KRIX 1 Fab fragments for fVIII .

To determine whether KRIX 1 was representative of the polyclonal antibodies from the donor, a competitive assay was used. The binding of biotinylated KRIX 1 to insolubilised fVIII was measured in presence of increasing concentrations of either KRIX 1, polyclonal IgG from the donor, or control polyclonal IgG. IgG from the donor dose-dependently inhibited KRIX 1 binding to fVIII. The concentration of KRIX 1 and IgG from the donor inhibiting 50% of biotinylated KRIX 1 on fVIII were of 0.3 µg/ml and of 170 µg/ml µg/ml, respectively, whereas no inhibition was observed with the control IgG.

EXAMPLE 4 production of monoclonal antibodies derived from hemophilia A patients. and which bind to the factor VIII and von Willebrand factor complex.

Alternatively, antibodies which reduce the release rate of factor VIII from von Willebrand factor are identified as follows. Polystyrene microtitration plates are coated with a specific antibody to von Willebrand factor (vWF). A solution of biotinylated recombinant factor VIII (0.5 microg/mL) complexed to vWF (5 microg/mL) is mixed with various concentrations of IgG from a donor (Fig. 5 solid squares), e.g. the same patient as described above (from which KRIX 1 was derived), MoAb4H1D7, or of IgG from a non-hemophiliac subject (Fig. 5 solid triangles). IgG at the indicated concentrations was added to microtitration plates coated with a mouse antibody, MoAb4H1D7 against von Willebrand factor for an incubation of two hours at room temperature. After washing. factor VIII was activated by thrombin during two minutes at 37°C. Factor VIII bound to vWF was detected by addition of avidine peroxydase. Controls included the detection of bound biotinylated factor VIII in the absence of thrombin digestion (OD450=460+47.7SD) and of biotinylated recombinant factor VIII after thrombin digestion in the absence of antibody (OD450=160+16.0SD).

Results of these experiments are shown in Fig. 5 for polyclonal antibodies. On this figure, the average values as well as the standard deviation of triplicates are indicated. Fig. 5 clearly shows that a significantly higher proportion of activated factor VIII remains bound to the plate in the presence of increasing concentrations of the antibody, i.e. it demonstrates a reduction of the dissociation of activated factor VIII from von Willebrand factor (vWF) in the presence of an inhibitor antibody recognizing factor VIII bound to vWF. Monoclonal antibodies have been obtained from these polyclonal antibodies in accordance with the methods described in this invention. These antibodies exhibit the "plateau" effect at molar excess as described above with respect to the KRIX 1 antibody thus indicating that the present invention may be extended to monoclonal antibodies and fragments and derivatives thereof which bind to complexes.

EXAMPLE 5: Sequencing of antibody variable domains

Sequencing of antibody variable domains was carried out as follows. The

isolation of RNA from EBV-immortalized human B-cell lines was performed using TRIzol Reagent according to the manufacturer's instructions (Life Technologies). CDNA was synthesized with the SuperScript preamplification system for first-strand cDNA synthesis. The cDNA encoding the heavy chain variable region genes (V_H) was amplified by polymerase chain reaction (PCR) using primers specific for the leader sequence of the V_H families and for the first exon of the C_γ region, as described. (Bakkus, heirman, Van-Riet, Van-Camp, Thielmans: Evidence that multiple myeloma Ig heavy chain VDJ genes contain somatic mutations but show no intraclonal variation, Blood, 80:2326, 1992) Annealing was performed at 60°C for 40 PCR cycles. PCR products of the appropriate size (460 bp) were isolated from 1.5% agarose gel and cloned using the TA Cloning Kit (Invitrogen BV, Leek. The Netherlands). A PCR screening using couples of primers corresponding to the V_H gene family of interest was performed on cultures of randomly selected colonies. Plasmid DNA from positive colonies were isolated using Wizard Plus Minipreps (Promega, Menlo Park, CA) and sequenced in both directions with Sequenase (US Biochemical,Cleveland. OH), according to the manufacturer's instructions. Analysis of the variable gene sequences was made using the V BASE Sequence Directory (Tomlinson et al, MRC Centre for Protein Engineering, Cambridge, UK).

The complete sequences of the V_H and V_L of the antibody BO 2C11 described in example 1 were submitted to the EMBL Nucleotide Sequence Database under the accession numbers AJ224083 and AJ224084, respectively. The complete sequence of the V_H and V_L of the KRIX 1 antibody described in Example 1 were submitted to the EMBL Nucleotide Sequence Database under the accession numbers AJ009732 and AJ009733.

The amino acid sequences in Figs. 6 and 7 define the VH and VL regions of the antibody BO 2C11 including the three CDR's 1-3 for each of the short and long chains. Also given are the polynucleotide sequences which encode for these regions.

The amino acid sequences in Figs. 8 and 9 define the VH and VL regions of the antibody KRIX-1 including the three CDR's 1-3 for each of the short and long chains. Also given are the polynucleotide sequences which encode for these regions.

CLAIMS

1. Cell line named KRIX 1 deposited with the Belgian Coordinated Collections of Micro-organisms, under accession number LMBP 5089CB.

2. Cell line producing human monoclonal antibodies having a reactivity substantially identical to that of the human monoclonal antibodies obtained from the cell line of claim 1.

3. A ligand having the capacity of only partially inactivating a factor or a complex of two or more factors involved in hemostasis when the ligand is in a physiological excess by binding to a site of the said factor or the said complex.

4. The ligand of claim 3, wherein the binding site of the ligand is not directly or substantially involved in a physiological interaction of the said factor or said complex.

5. The ligand according to claim 3, wherein the factor or the complex is involved in the coagulation cascade of blood

6. The ligand according to claim 5, wherein the factor is factor VIII or factor VIII is involved in the complex.

7. The ligand according to claim 6, having the capacity of inactivating the co-factor activity of factor VIII by interfering with a proteolytic cleavage site or the von Willebrand factor or the tenase complex reaction or by inducing a three-dimensional conformational change in factor VIII or by targeting a domain of factor VIII, in particular the C1 domain of factor VIII, or by targeting the factor VIII-von Willebrand factor complex.

8. The ligand according to claim 3, being a human monoclonal antibody obtainable from the cell lines of claim 1 or claim 2.

9. The ligand according to claim 8, being of class IgG.

10. The ligand according to claim 3, being able to recognize epitopes located in the C1 domain of factor VIII.

11. The ligand according to claim 8, being able to recognize epitopes located in the C1 domain of factor VIII.

12. A ligand according to claim 9, being able to recognize epitopes located in the C1 domain of factor VIII.

13. The ligand according to claim 3, having substantially the same characteristics as a ligand according to claim 8 and being a monoclonal antibody produced by on purpose immunization in animals, preferably in mouse.

14. A humanized monoclonal antibody obtainable from the monoclonal antibody of claim 13.

15. An antigen-binding fragment Fab, Fab' or $F(ab')_2$, a complement-binding fragment F_C, a complementarity determining region, a soluble or membrane-anchored single-chain variable part, a single variable domain or a derivative of a monoclonal antibody according to claim 8.

16. An antigen-binding fragment Fab, Fab' or $F(ab')_2$, a complement-binding fragment F_C, a complementarity determining region, a soluble or membrane-anchored single-chain variable part, a single variable domain or a derivative of a monoclonal antibody according to claim 11.

17. An antigen-binding fragment Fab, Fab' or $F(ab')_2$, a complement-binding fragment F_C, a complementarity determining region, a soluble or membrane-anchored single-chain variable part, a single variable domain or a derivative of a humanized monoclonal antibody according to claim 14.

18. The ligand according to claim 3, having the capacity of binding to a site which is at a predetermined distance of a physiologically functional site of the said factor.

19. A pharmaceutical composition for the prevention or treatment of disorders of hemostasis and resulting pathologic conditions in humans or animals, comprising a ligand according to claim 3 or claim 6 or claim 7 or claim 18, in admixture with a pharmaceutically acceptable carrier.

20. A pharmaceutical composition for the prevention or treatment of disorders of hemostasis and resulting thrombotic pathologic conditions in humans or animals, comprising a human monoclonal antibody according to claim 8, in admixture with a pharmaceutically acceptable carrier.

21. A pharmaceutical composition for the prevention or treatment of disorders of the coagulation cascade and resulting thrombotic pathologic conditions in humans or animals, comprising a human monoclonal antibody according to claim 9, in admixture with a pharmaceutically acceptable carrier.

22. A pharmaceutical composition for the prevention or treatment of disorders hemostasis and resulting pathologic conditions in humans, comprising a human monoclonal antibody according to claim 11, in admixture with a pharmaceutically acceptable carrier.

23. A pharmaceutical composition for the prevention or treatment of disorders of hemostasis and resulting pathologic conditions in humans, comprising a human monoclonal antibody according to claim 12, in admixture with a pharmaceutically acceptable carrier.

24. A pharmaceutical composition for the prevention or treatment of disorders hemostasis and resulting pathologic conditions in humans or animals, comprising a ligand according to claim 13, in admixture with a pharmaceutically acceptable carrier.

25. A pharmaceutical composition for the prevention or treatment of disorders of hemostasis and resulting pathologic conditions in humans, comprising a humanized monoclonal antibody according to claim 14, in admixture with a pharmaceutically acceptable carrier.

26. A pharmaceutical composition according to claim 19, wherein partial inactivation of the factor involved in hemostasis is an at most 99% inactivation or an at most 95%, an at most 90% or an at most 80% inactivation.

27. A pharmaceutical composition according to claim 26, wherein partial inactivation of the factor involved in hemostasis is an at least 50% or an at least 60% or an at least 70% inactivation.

28. A pharmaceutical composition according to claim 19, further comprising a thrombolytic agent.

29. A pharmaceutical composition for the prevention or treatment of disorders of hemostasis and resulting pathologic conditions in humans, comprising an antigen-binding fragment Fab, Fab' or F(ab')2 or a complement-binding fragment F_c or a complementarity determining region or a soluble or membrane-anchored single-chain variable part or a single variable domain or a derivative according to claim 15 or claim 16 or claim 17, in admixture with a pharmaceutically acceptable carrier.

30. A method of treatment and/or prevention of a disorder of hemostasis, coagulation disorder or thrombotic pathologic condition or attenuation of coagulation in a human or ina an animal, comprising administering to a patient in need of such treatment or prevention or attenuation of coagulation a therapeutically effective amount of a ligand according to claim 3 or claim 6 or claim 7 or claim 18.

31. A method of treatment and/or prevention of a hemostasis disorder, coagulation disorder or thrombotic pathologic condition or attenuation of coagulation in a human, comprising administering to a patient in need of such treatment or prevention or attenuation of coagulation a therapeutically effective amount of a human monoclonal antibody according to claim 8.

32. A method of treatment and/or prevention of a hemostasis disorder, coagulation disorder

or thrombotic pathologic condition or attenuation of coagulation in a human, comprising administering to a patient in need of such treatment or prevention or attenuation of coagulation a therapeutically effective amount of a human monoclonal antibody according to claim 9.

33. A method of treatment and/or prevention of a hemostasis disorder, coagulation disorder or thrombotic pathologic condition or attenuation of coagulation in a human, comprising administering to a patient in need of such treatment or prevention or attenuation of coagulation a therapeutically effective amount of a human monoclonal antibody according to claim 11.

34. A method of treatment and/or prevention of a hemostasis disorder, coagulation disord r or thrombotic pathologic condition or attenuation of coagulation in a human, comprising administering to a patient in need of such treatment or prevention or attenuation of coagulation a therapeutically effective amount of a human monoclonal antibody according to claim 12.

35. A method of treatment and/or prevention of a hemostasis disorder, coagulation disorder or thrombotic pathologic condition or attenuation of coagulation in a human or in an animal, comprising administering to a patient in need of such treatment or prevention or attenuation of coagulation a therapeutically effective amount of a ligand according to claim 13.

36. A method of treatment and/or prevention of hemostasis disorder, a coagulation disorder or thrombotic pathologic condition or attenuation of coagulation in a human, comprising administering to a patient in need of such treatment or prevention or attenuation of coagulation a therapeutically effective amount of a humanized monoclonal antibody according to claim 14.

37. A method of treatment and/or prevention of a hemostasis disorder, coagulation disorder or thrombotic pathologic condition or attenuation of coagulation in a human, comprising administering to a patient in need of such treatment or prevention or attenuation

of coagulation a therapeutically effective amount of an antigen-binding fragment Fab, Fab' or $F(ab')_2$ or a complement-binding fragment F_c or a complementarity determining region or a soluble or membrane-anchored single-chain variable part or a single variable domain, or a derivative according to claim 15 or claim 16 or claim 17.

38. A method according to claim 30, wherein the thrombotic pathologic condition is selected from intravascular coagulation, arterial thrombosis, arterial restenosis, venous thrombosis and arteriosclerosis.

39. A method according to claim 37, wherein the thrombotic pathologic condition is selected from intravascular coagulation, arterial, thrombosis, arterial restenosis, venous thrombosis and arteriosclerosis.

40. A method according to claim 30, wherein the ligand is provided to a patient by oral, intranasal, subcutaneous, intramuscular, intradermal, intravenous, intraarterial or parenteral administration or by catheterization.

41. A method according to claim 37, wherein the antigen-binding fragment Fab, Fab' or $F(ab')_2$ or a complement-binding fragment F_c or a complementarity determining region or a soluble or membrane-anchored single-chain variable part or a single variable domain or a derivative is provided to a patient by oral, intranasal, subcutaneous, intramuscular, intradermal, intravenous, intraarterial or parenteral administration or by catheterization.

42. A method according to claim 30, further comprising sequentially administrating to the patient a therapeutically effective amount of a thrombolytic agent.

43. A method according to claim 37, comprising further sequentially administrating to the patient a therapeutically effective amount of a thrombolytic agent.

44. A human or a reshaped human monoclonal antibody which binds to and at least partially inactivates factor VIII or a complex including factor VIII and comprises only elements derived from the repertoire of human antibodies.

45. The antibody according to claim 44, wherein the anitibody interfers with a proteolytic cleavage site or the von Willebrand factor or the tenase complex reaction or by inducing a three-dimensional conformational change in factor VIII or by targeting a domain of factor VIII, in particular the C1 domain of factor VIII, or by trageting the factor VIII-von Willebrand factor complex.

46. A polynucleotide encoding for an antigen-binding fragment Fab, Fab' or $F(ab')_2$ or a complement-binding fragment F_c or a complementarity determining region or a soluble or membrane-anchored single-chain variable part or a single variable domain, or a derivative according to claim 15 or claim 16 or claim 17.

47. A method of obtaining monoclonal antibodies, comprising the steps of:
1. providing a mammal having a modified and at least partially functional physiologically active protein, the modification being with respect to a wild type protein and lying in a domain of the protein;
2. administering the wild type protein to the mammal to illicit an immune response; and
3. selecting B-lymphocytes from the mammal which produce antibodies which at least partially inactivate the wild type protein.

48. The method according to claim 46, wherein the protein is a factor or factors in hemostasis.

49. The method according to claim 47, wherein the factor is factor VIII or a complex including factor VIII.

ABSTRACT

The present invention comprises ligands and methods of manufacture thereof as well as pharmaceutical preparations including the ligands. The ligands may be human or humanized monoclonal antibodies and fragments, derivatives and homologs thereof. These may exhibit an unforeseen "plateau effect", i.e. the achievement of only a partial inactivation of a factor involved in hemostasis, in particular in the coagulation cascade, either individually or in combination even in molar excess. The ligands may bind to a factor or a complex of factors resulting in only partially impairing the function of a physiologically functional site of the said factor or factor complex even in molar excess. This makes the ligands particularly suitable for treating coagulation disorders and resulting thrombotic pathologic conditions while minimizing the risk of bleeding. Particularly useful is a property of ligands in accordance with the present invention to allow some physiological function of the affected site even when the ligand is in molar excess. The ligands may be anti-factor VIII antibodies or antibodies against a factor VIII complex, in particular human or human hybrid monoclonal antibodies which bind to factor VIII or a factor VIII complex and at least partially inhibit the activity of factor VIII.

Fig. 3

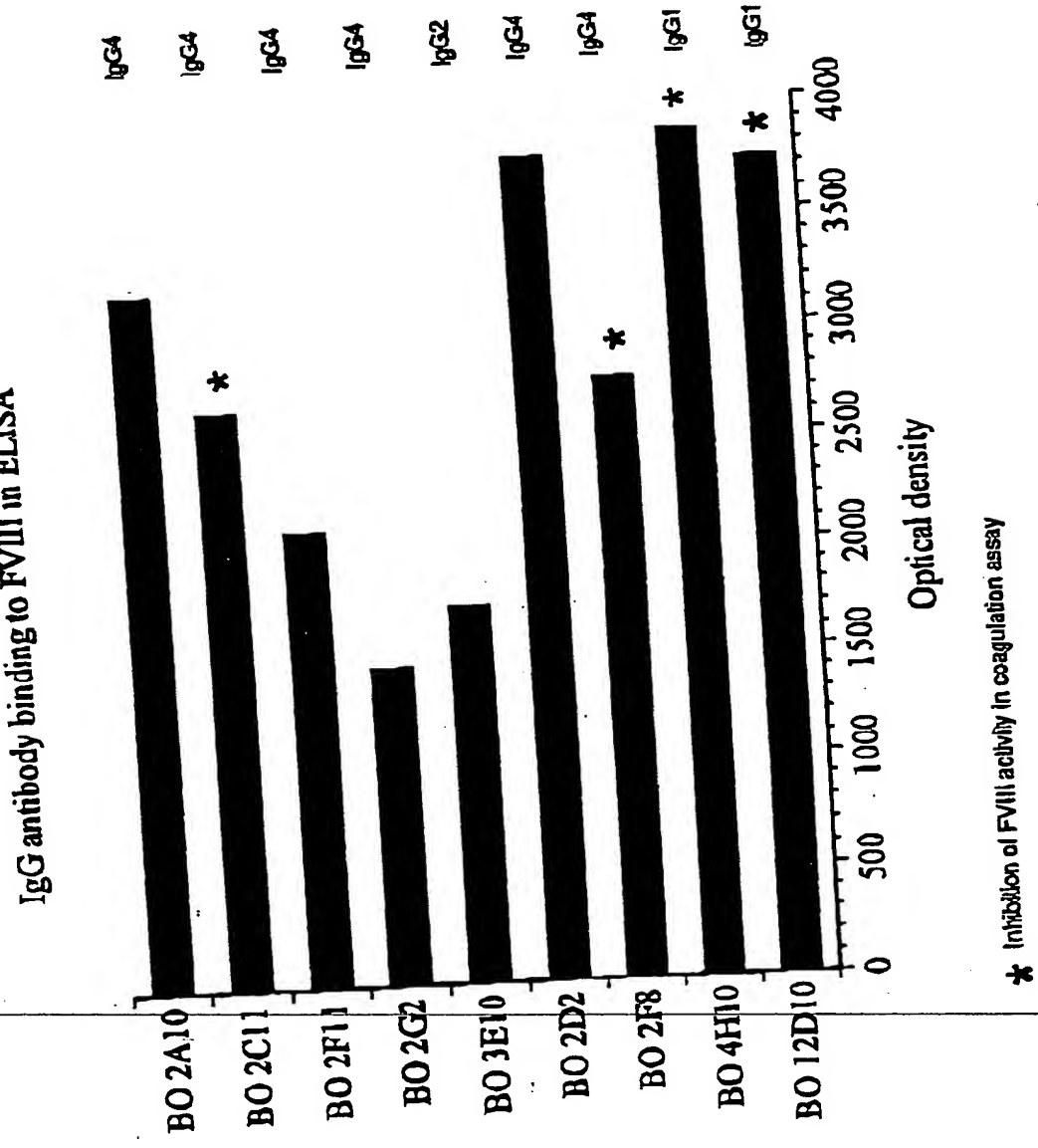
IgG antibody binding to FVIII in ELISA
IgG4
IgG4
IgG4
IgG4
IgG2
IgG4
IgG4
IgG1
IgG1
*
*
*
*
0
500
1000
1500
2000
2500
3000
3500
4000
Optical density
BO 2A10
BO 2C11
BO 2F11
BO 2G2
BO 3E10
BO 2D2
BO 2F8
BO 4H10
BO 12D10
* Inhibition of FVIII activity in coagulation assay

Fig. 1

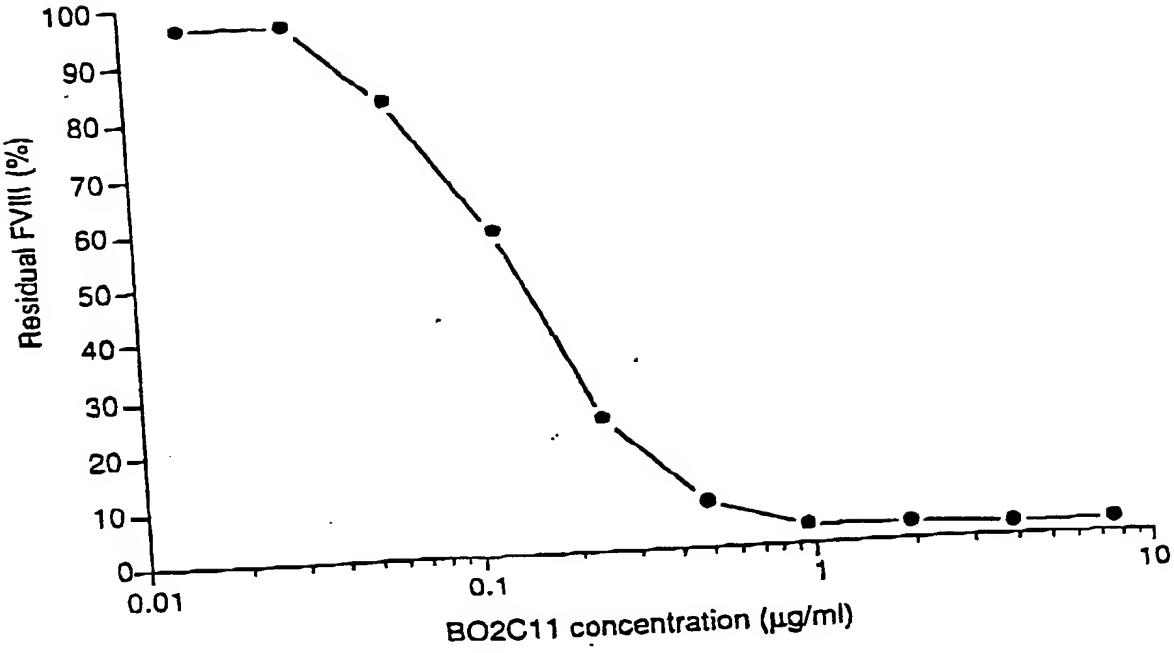
Residual FVIII (%)
100
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1
10
BO2C11 concentration (µg/ml)

Fig. 2

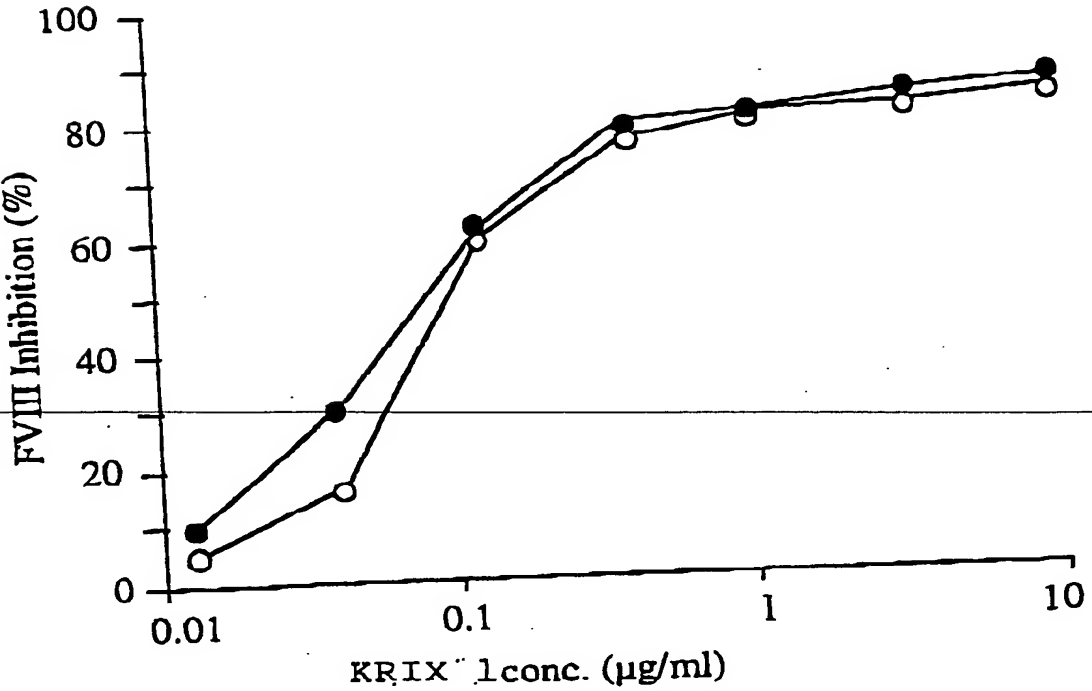
FVIII Inhibition (%)
100
80
60
40
20
0
0.01
0.1
1
10
KRIX 1conc. (µg/ml)

Fig. 3

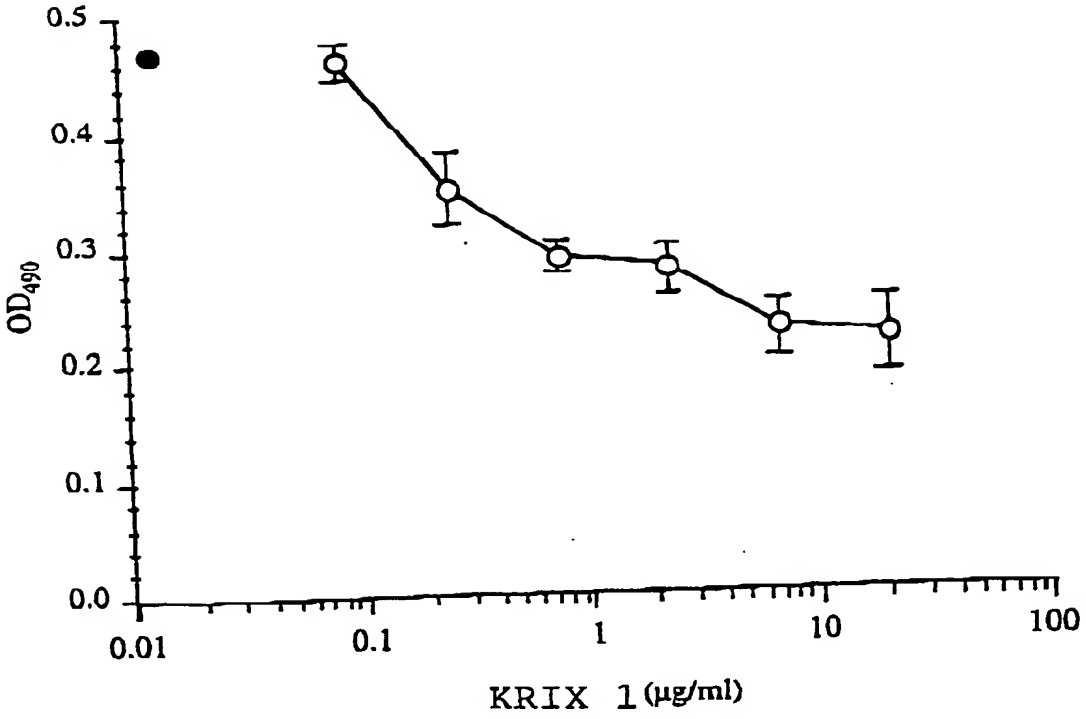
0.5
0.4
0.3
0.2
0.1
0.0
OD_{490}
0.01
0.1
1
10
100
KRIX 1 (µg/ml)

Fig. 4

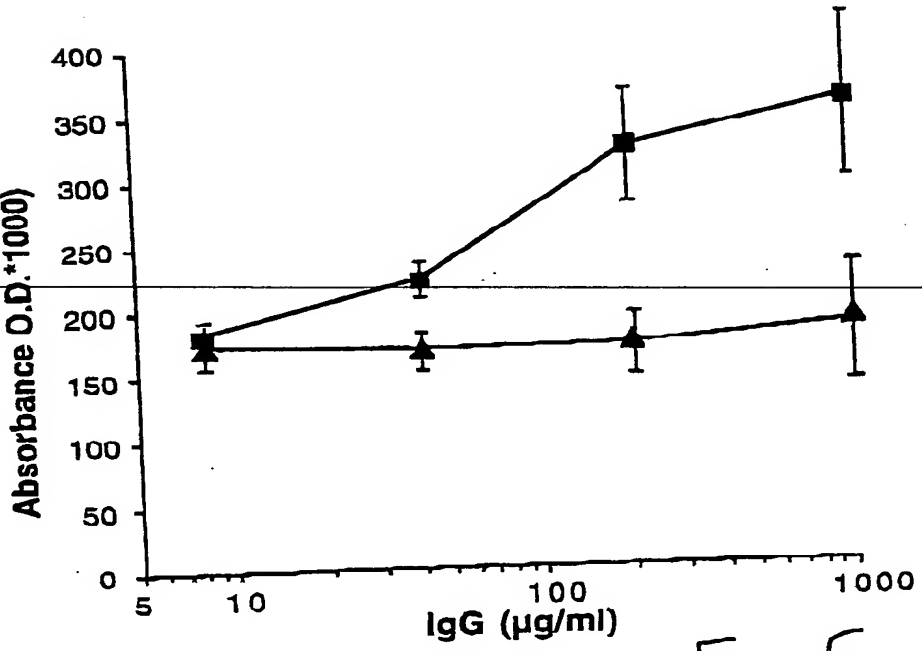
400
350
300
250
200
150
100
50
0
Absorbance O.D.*1000)
5
10
100
1000
IgG (µg/ml)

Fig. 5

VH BO 2C11

```
                                                    31/11
1/1
atg gac tgg acc tgg agg atc ctc ttc ttg gtg gca gca gct aca ggc acc cac gcc cag
Met asp trp thr trp arg ile leu phe leu val ala ala ala thr gly thr his ala gln

                                                    91/31
61/21
gtc caa ctg gta cag tct ggg gct gag gtg aag aag cct ggg gcc tca gtg aag gtc tcc
val gln leu val gln ser gly ala glu val lys lys pro gly ala ser val lys val ser

                                                    151/51
121/41
tgc aag gtt tcc gga tac acc ctc act gaa tta ccc gtg cac tgg gtc gga cag gct cct
cys lys val ser gly tyr thr leu thr glu leu pro val his trp val gly gln ala pro
                  <---------------CDR 1----------------->

                                                    211/71
181/61
gga aaa ggg ctt gag tgg gtg gga agt ttt gat cct gaa agt gga gaa tca atc tac gca
gly lys gly leu glu trp val gly ser phe asp pro glu ser gly glu ser ile tyr ala
                                <-------------------------CDR 2-----------------

                                                    271/91
241/81
cgg gag ttc cag ggc agc gtc acc atg acc gcg gac aca tct aca gac ata gcc tac atg
arg glu phe gln gly ser val thr met thr ala asp thr ser thr asp ile ala tyr met
---------------------->

                                                    331/111
301/101
gag ctg agc agc ctg aga tct gac gac acg gcc gtg tat tac tgt gca gtc cct gac cct
glu leu ser ser leu arg ser asp asp thr ala val tyr tyr cys ala val pro asp pro
                                                    | <---------- - - - - - -

                                                    391/131
361/121
gat gct ttt gat atc tgg ggc caa ggg aca atg gtc acc gtc tct tca gcc tcc acc aag
asp ala phe asp ile trp gly gln gly thr met val thr val ser ser ala ser thr lys
--CDR 3---------->

421/141
ggc cca tcg gtc ttc ccc ctg gga tcc cgt
gly pro ser val phe pro leu gly ser arg
```

Fig. 6

VL BO 2C11

```
1/1                                                  31/11
atg gaa acc cca gct cag ctt ctc ttc ctc ctg cta ctc tgg ctc cca gat acc acc gga
Met glu thr pro ala gln leu leu phe leu leu leu leu trp leu pro asp thr thr gly

61/21                                                91/31
gaa att gcg ttg acg cag tct cca ggc acc ctg tct ttg tct cca ggg gaa aga gcc acc
glu ile ala leu thr gln ser pro gly thr leu ser leu ser pro gly glu arg ala thr

121/41                                               151/51
ctc tcc tgc agg gcc agt cag agt ttt agc agc agc tac tta gcc tgg tat cag cag aaa
leu ser cys arg ala ser gln ser phe ser ser ser tyr leu ala trp tyr gln gln lys
          <----------------CDR 1---------------------->

181/61                                               211/71
cct ggc cag gct ccc agg ctc ctc atc tat ggt gca tcc acc agg gcc act ggc atc cca
pro gly gln ala pro arg leu leu ile tyr gly ala ser thr arg ala thr gly ile pro
                                    <---------CDR 2----------->

241/81                                               271/91
gac agg ttc agt ggc agt ggg tct ggg aca gac ttc act ctc acc atc agc aga ctg gag
asp arg phe ser gly ser gly ser gly thr asp phe thr leu thr ile ser arg leu glu

301/101                                              331/111
cct gaa gat ttt gca gtg tat tac tgt cag aag tat ggt acg tca gcg atc acc ttc ggg
pro glu asp phe ala val tyr tyr cys gln lys tyr gly thr ser ala ile thr phe gly
                                  <-------------CDR 3--------------->

361/121                                              391/131
caa ggg aca cga ctg gag att aaa gga act gtg gct gca cca tct gtc ttc atc ttc ccg
gln gly thr arg leu glu ile lys gly thr val ala ala pro ser val phe ile phe pro

421/141
cca tct
pro ser
```

Fig. 7

VH KRIX 1

```
                                                                  31/11
1/1
ATG AAA CAC CTG TGG TTC TTC CTC CTG CTG GTG GCA GCT CCC AGA TGG GTC CTG TCC CAG
Met lys his leu trp phe phe leu leu leu val ala ala pro arg trp val leu ser gln

                                                                  91/31
61/21
CTG CAC CTG CAA GAG TCC GGC TCA GGA CTG GTG AAG CCT TCA CAG ACC CTG TCC CTC ACC
leu his leu gln glu ser gly ser gly leu val lys pro ser gln thr leu ser leu thr

                                                                  151/51
121/41
TGC GTT GTC TCT GGT GAC TCC ATC AGC AGT GAT GGA TAC TCC TGG AGC TGG ATC CGG CAG
cys val val ser gly asp ser ile ser ser asp gly tyr ser trp ser trp ile arg gln
                <--------------------CDR 1---------------------->

                                                                  211/71
181/61
CCA CCA GGG AAG GGC CTG GAG TGG ATT GGG TAC ATC TTT CAT AGT GGG AGC ACC TAC TGC
pro pro gly lys gly leu glu trp ile gly tyr ile phe his ser gly ser thr tyr cys
                                    <------------------------------CDR 2-- - - -

                                                                  271/91
241/81
AAC CCG TCT CTC AAG AGT CGA GTC ACC ATA TCA ATA GAC AGG GTC ATG AAC CGG TTC TCC
asn pro ser leu lys ser arg val thr ile ser ile asp arg val met asn arg phe ser
------------------------->

                                                                  331/111
301/101
CTG AAG TTG AGC TCT GTG ACC GCC GCG GAC ACG GCC GTC TAT TAC TGT GCC AGA GAT AGT
leu lys leu ser ser val thr ala ala asp thr ala val tyr tyr cys ala arg asp ser
                                                          <------- - - - - -

                                                                  391/131
361/121
GGT GGT CTT TTT CAA TTA GAC TAC TGG GGC CAG GGA ACC CTG GTC ACC GTC TCC TCA GCC
gly gly leu phe gln leu asp tyr trp gly gln gly thr leu val thr val ser ser ala
----CDR 3---------------------->

                                                                  451/151
421/141
TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GGA TCC CGT
ser thr lys gly pro ser val phe pro leu gly ser arg
```

Fig. 8

VL KRIX 1

```
1/1                                                    31/11
ATG GAA ACC CCA GCT CAG CTT CTC TTC CTC CTG CTA CTC TGG CTC CCA GAT ACC ACC GGA
Met glu thr pro ala gln leu leu phe leu leu leu leu trp leu pro asp thr thr gly

61/21                                                  91/31
GAA ATT GTG TTG ACG CAG TTC CCA GGC ACC CTG TCT TTG TCT CCA GGG GAA AGA GCC ACC
glu ile val leu thr gln phe pro gly thr leu ser leu ser pro gly glu arg ala thr

121/41                                                 151/51
CTC TCC TGC AGG GCC AGT CAG AGT GTT GCC AGC GCC TAC TTA GCC TGG TAC CAG CAA AAA
leu ser cys arg ala ser gln ser val ala ser ala tyr leu ala trp tyr gln gln lys
          <-------------------CDR 1---------------------->

181/61                                                 211/71
CCT GGC CAG GCT CCC AGG CTC CTC ATC TAT GGT GCA TCC AGT AGG GCC ACC GAC ATC CCA
pro gly gln ala pro arg leu leu ile tyr gly ala ser ser arg ala thr asp ile pro
                                  <---------CDR 2----------->

241/81                                                 271/91
CAC AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACT CTC ACC ATC AGC AGA CTG GAG
his arg phe ser gly ser gly ser gly thr asp phe thr leu thr ile ser arg leu glu

301/101                                                331/111
CCT GAA GAT TTT GCA GTG TAC TAC TGT CAG CAA TAT GGT ACC TCA GCC TTA CTC ACT TTC
pro glu asp phe ala val tyr tyr cys gln gln tyr gly thr ser ala leu leu thr phe
                               <----------------CDR 3---------------->

361/121                                                391/131
GGC GGA GGG ACC AAG GTG GAG ATC AAA CGA ACT GTG GCT GCA CCA TCT GTC TTC ATC TTC
gly gly gly thr lys val glu ile lys arg thr val ala ala pro ser val phe ile phe

421/141
CCG CCA TCT
pro pro ser
```

Fig. 9